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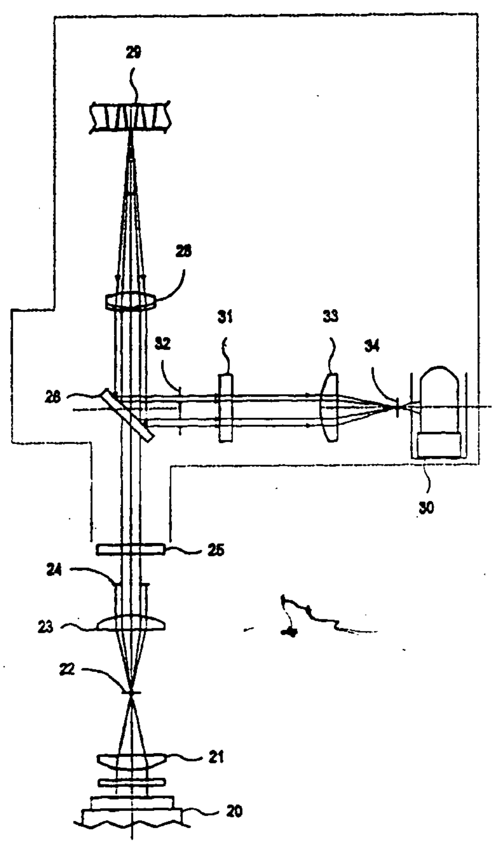
(54) Title: MEASUREMENT OF FLUORESCENCE

(57) Abstract

A method and apparatus for assaying samples in a test well (39) having opaque walls and a transparent bottom by exciting the samples with a beam of light of a predetermined wavelength (25) and measuring (30) the light emitted by fluorescence from the sample. The exciting light (25) and the emitted light (31) pass through the transparent bottom of the test well (29) which may be polystyrene having a thickness no greater than about 0.010 inches or glass or quartz glass.



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MEASUREMENT OF FLUORESCENCE

BACKGROUND OF THE INVENTION

This invention is related generally to the measurement of liquid samples by means of fluorescence. In a preferred embodiment, it relates to the measurement of samples which have been placed in microplates having multiple wells with transparent bottoms. Typical plates were disclosed in US Patent 5,319,436.

Measurement of samples by fluorescence has been the subject of a number of patents, notably US 5,018,866 and US 4,501,970. In the '866 patent improved results were obtained by directing a focused beam of light into a sample well and recovering the light emitted by fluorescence through the use of an elliptical reflector surrounding the sample well which focused the light onto a photodetector. A related use of an elliptical shape to reflect emitted light is found in US 3,946,239. In the '970 patent a system is disclosed which focuses a beam of light on a spot within a sample well and focuses the emitted light onto a photodetector. The exciting beam enters and the emitted light leaves the sample well from the top, thereby avoiding the problems associated with the use of plastic materials which have a native fluorescence, as discussed in the background section of the '970 patent.

The present inventors have addressed the problem of using a plastic test plate containing multiple sample wells in measuring samples by means of fluorescence, as will be seen in the description which follows.

SUMMARY OF THE INVENTION

In one aspect, the invention is a method of assaying samples of liquids by fluorescence. A sample is placed in a sample well having opaque sides and a transparent bottom. A beam of light is focused on the sample through the transparent bottom, causing it to fluoresce, and then the resulting emitted light passes out through the transparent bottom and is directed to a photodetector. The desired property of the sample is correlated with the output of the photodetector. In the typical case, fluorescence is produced by a dye selected to interact with the sample, thus permitting measurement of the sample by excitation of the dye.

The exciting light beam preferably is focused in the middle of the sample well and contact with the walls of the sample well is avoided. The light emanating from the bottom of the sample well is focused to minimize the amount entering the photodetector

which has been scattered from the walls or generated by the native fluorescence of the well materials. The transparent bottom of the sample well, the "window", may be made of various clear materials having a low response to the exciting light, that is, they should exhibit a low native fluorescence. Preferably glass, or quartz glass will be used. When plastic materials such as polystyrene are used the thickness of the bottom window should be minimized. For polystyrene the thickness preferably will be no greater than about 250 μm (0.010 inches).

In another aspect, the invention is a microplate for assaying a liquid sample by determining the amount of fluorescence resulting from the contact of an exciting light beam. The microplate contains a multiplicity of sample wells for containing the liquid samples and has opaque walls and a transparent bottom (a "window"). The exciting light beam is focused on the sample from below, through the transparent bottom window and the light emitted by fluorescence exits through the bottom and is focused on a photodetector for measurement. The transparent bottom window will have a relatively low native fluorescence. Glass, or quartz glass provide a better signal-to-noise ratio, but polystyrene may be used if the thickness is no greater than about 250 μm (0.010 inches).

In still another aspect of the invention, an instrument having multiple lenses and apertures provides a beam of light at a suitable wavelength to excite fluorescence in a sample and directs light emitted from the samples via lenses and apertures to a photodetector.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an exploded view of a typical microplate.

Figures 2a-c show an optical system according to the invention.

Figures 3 and 4 are graphs of signal-to-noise ratio versus light emission wavelength for quartz and polystyrene cuvettes.

Figures 5 and 6 are alternate optical systems of the invention.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

MICROPLATES

The invention in its broad aspects could be applied to measurements made in various types of sample containers, each having sides which are opaque to the light used to excite fluorescence from a sample and a transparent bottom or window through which

the exciting light enters and the light emitted by fluorescence exits. The invention is particularly useful in making measurements of small samples in a multi-well tray (a microplate) such as those described in US Patent 5,319,436, which is incorporated by reference herein for a more complete description. A typical microplate 10 is shown disassembled in Figure 1, so that the bottom including the transparent windows is more clearly shown. The user normally receives the two pieces 11 and 13 fused together and ready to receive samples. Such microplates usually are arranged to have a 8 x 12 or 4 x 6 array of sample wells so that a large number of samples can be measured quickly. The upper portion 11 of the microplate 10 is molded of plastic with an array of cylindrical containers 12 forming the side walls of the sample wells. The lower portion 13 is a flat transparent plate which covers the bottom of the cylindrical containers and completes the sample wells. It should be noted that when the bottom plate is termed "transparent" that this refers to the ability of the material to pass light of the desired wavelength without significant attenuation. Similarly, "opaque" means that light of the wavelength being used does not pass through the material at all. The bottom of the sample well is a window 15 which is surrounded by raised portion 14 to isolate the sample wells optically.

The materials used for the microplates described in the '436 patent are typically rigid plastics such as those mentioned in the patent. Polystyrene is often used since it can withstand contact with many of the commonly analyzed materials and it is inexpensive so that after a single use it can be disposed of. Perhaps of more importance is its ability to bind biological materials such as cells, antibodies, receptors, enzymes and proteins.

Such microplates have been used to analyze radioactive samples. Detectors can be positioned above or below or in some cases, both above and below the sample well. When measurements are made by exciting the sample to cause it to fluoresce, the exciting light could be supplied from the top of the well, as is suggested in US Patent 4,501,970 mentioned above, thus avoiding attenuation of the light or native fluorescence of anything but the sample itself. However, when the exciting light beam is directed into the sample from below through the transparent bottom window, careful attention must be paid to the effects of the window, the sidewalls, the meniscus at the top of the sample and any other features of the sample and the well materials and geometry which could affect the exciting light or the emitted light which is produced by fluorescence. Thus, the

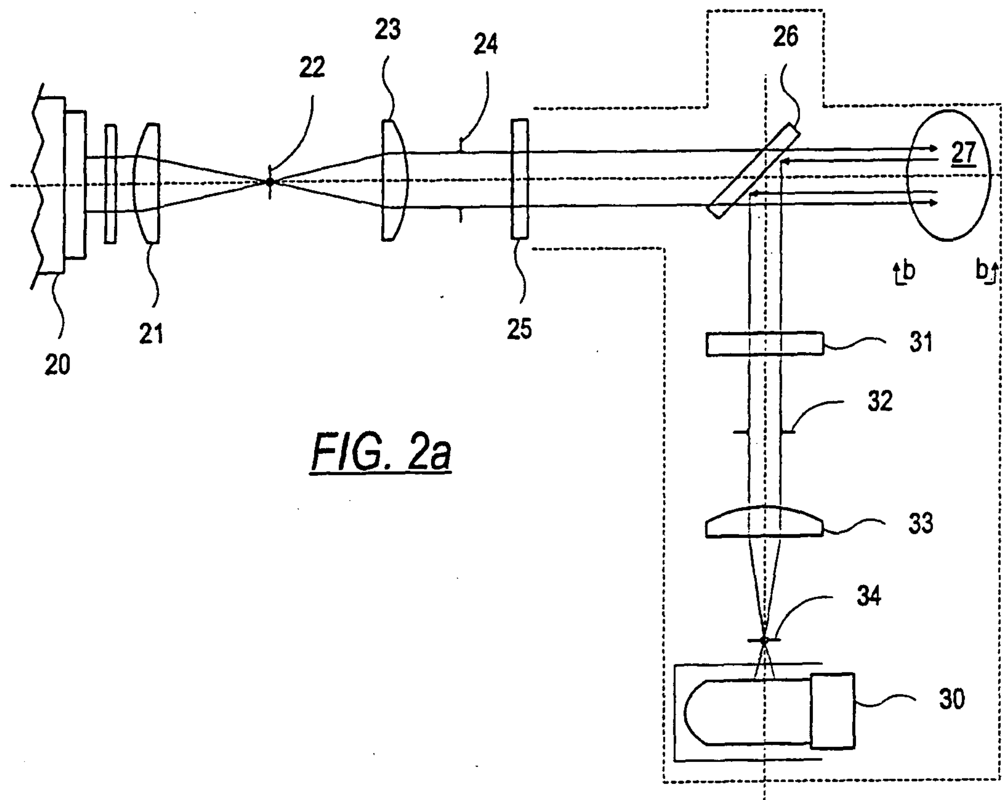


FIG. 2a

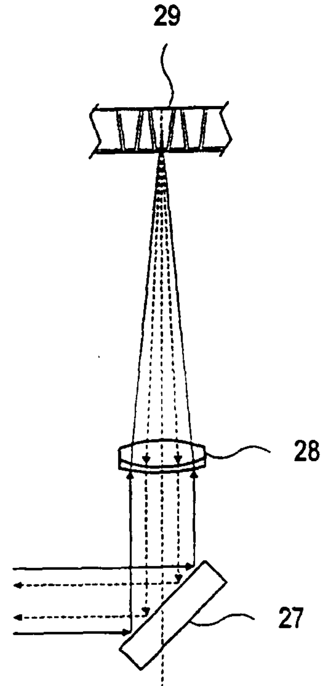


FIG. 2b

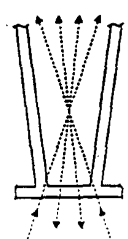


FIG. 2c

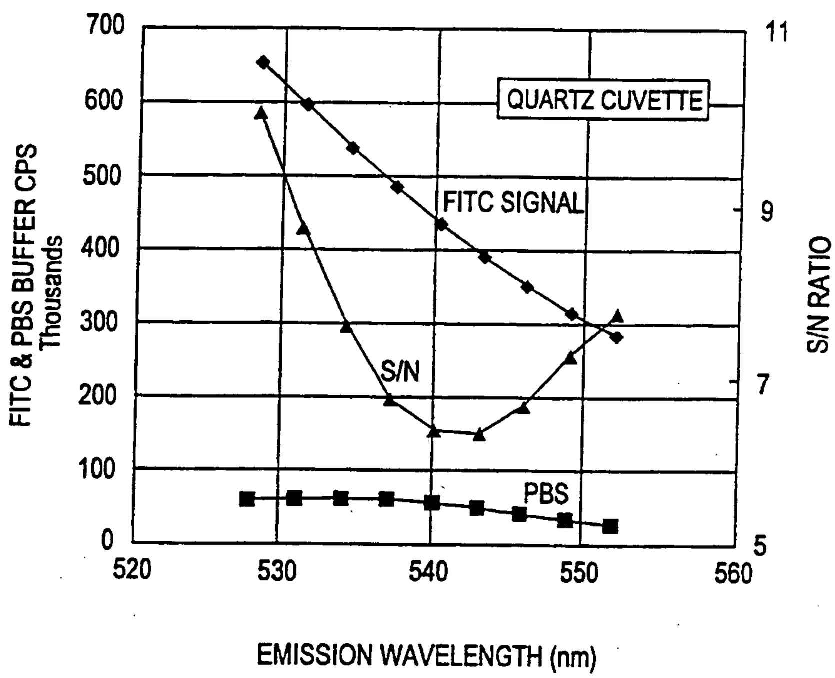
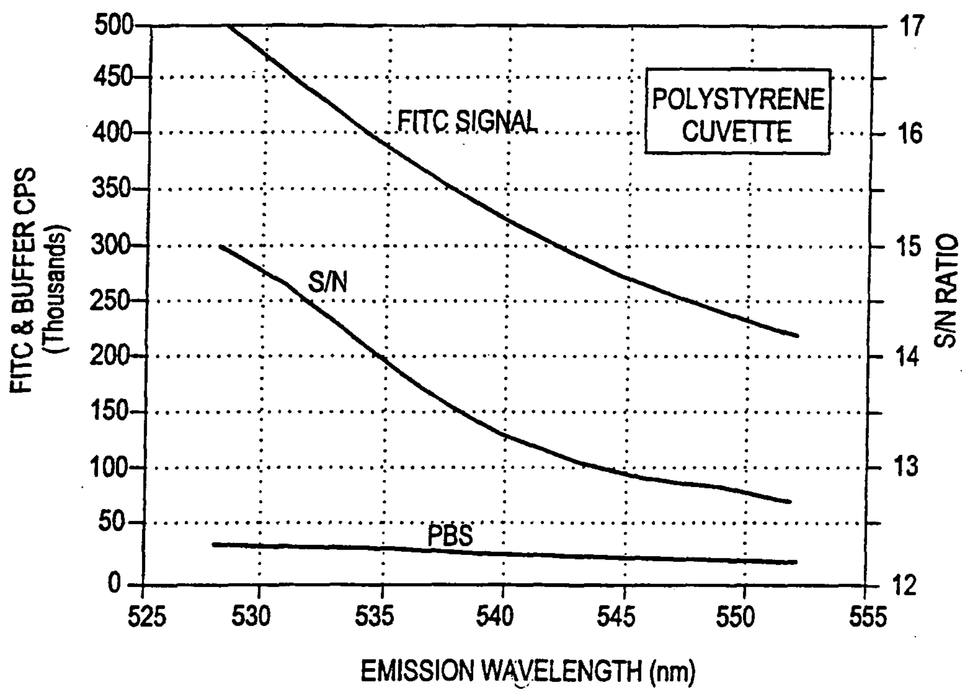

QUARTZ CUVETTE
FITC SIGNAL
S/N
PBS
FITC & PBS BUFFER CPS Thousands
S/N RATIO
EMISSION WAVELENGTH (nm)
0
100
200
300
400
500
600
700
5
7
9
11
520
530
540
550
560

FIG. 3



500
450
400
350
300
250
200
150
100
50
0
FITC & BUFFER CPS
(Thousands)
17
16
15
14
13
12
S/N RATIO
525
530
535
540
545
550
555
EMISSION WAVELENGTH (nm)
FITC SIGNAL
S/N
PBS
POLYSTYRENE
CUVETTE

FIG. 4

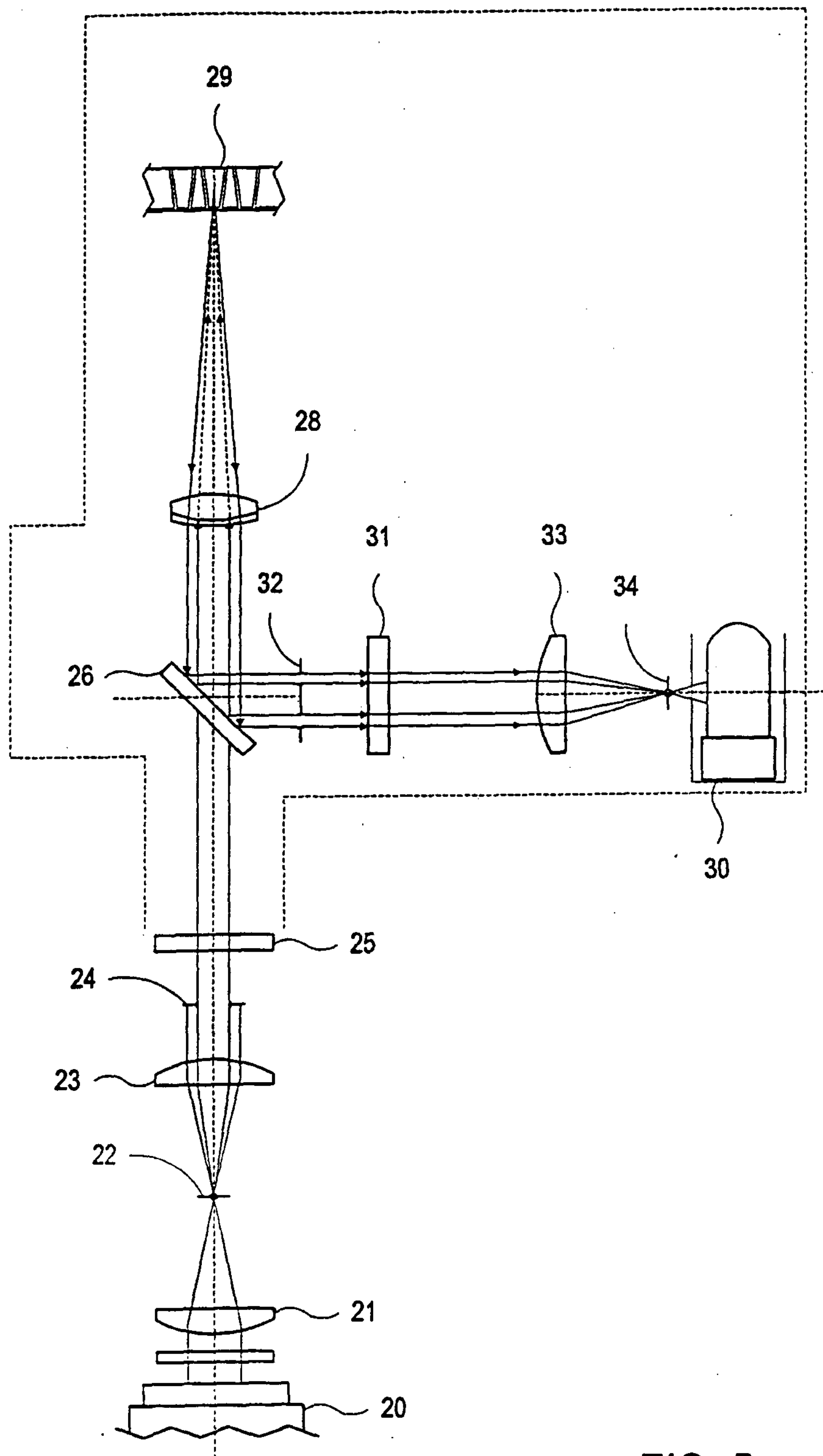


FIG. 5

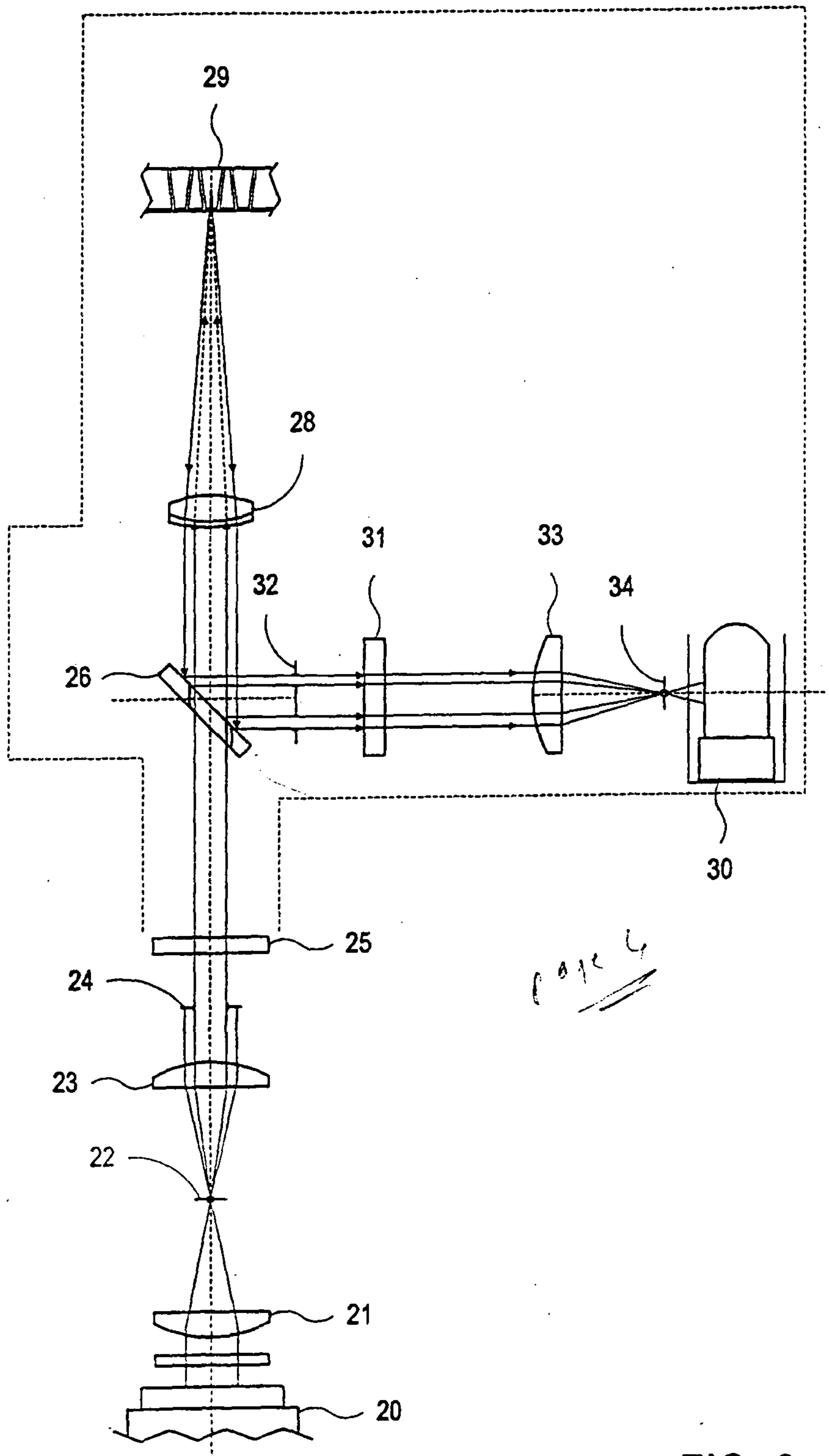


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06906

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :GO1N 21/64
US CL :356/417; 250/458.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 356/417; 250/458.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,018,866 A (OSTEN) 28 May 1991,(28-05-91),col.2 lines 20-21	1-18
Y	US 5,319,436 A (MANNS et al) 07 June 1994 (07-06-94) , see Fig.5	1-18
Y	US 4,501,970 A (NELSON) 26 February 1985 (26-02-85), col. 8, lines 20-25 and 35-40.	1-18
Y	US 5,355,215 A (SCHROEDER et al) 11 October 1994 (11-10-94), see Figs 1,2,6.	1-19
Y ------ A	US 5,578,818 A (KAIN et al) 26 November 1996 (26-11-96),see Fig.5.	19 -------- 20-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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system used for analyzing samples through the bottom of the sample wells must be arranged to obtain the optimum results and to produce the best possible signal-to-noise ratio (S/N).

OPTICAL SYSTEM

An optical system for use in the invention introduces a beam of light of a predetermined wavelength (the exciting light) through the transparent bottom of a sample well and transmits light emitted by fluorescence of a sample in the sample well from the bottom of the sample well to a photodector which measures the emitted light. The exciting light is columated and focused within the sample well by the optical system. The emitted light is collected and focused by the optical system so that other sources of light are minimized and the signal-to-noise ratio is maximized. In typical systems, selective optical filters are used to provide light having a wavelength which will cause the sample to fluoresce and to limit the wavelength of light transmitted to the photodetector to that emitted by the sample. It should be understood that by "wavelength" is meant a range of wavelengths within which a selected fluorescent dye can be excited or within which the emitted light is found. As will be seen below, the typical fluorescent dyes used with such optical systems are excited within a range centered at one wavelength and emit light at another longer wavelength.

Figure 2 illustrates an optical system which was used in making the measurements reported in the examples below. Other preferred arrangements for practical applications of the invention are shown in Figures 5 and 6. The source of the exciting light beam in the examples was a tungsten halogen lamp 20 operating at 10 volts. It produces light having wavelengths within the range of about 280 to 3000 nm. Other potentially useful light sources include for example, xenon, xenon arc, and quartz halogen lamps. This light must be focused and filtered to produce a columated beam having a predetermined center wavelength and limited to a narrow range about that center wavelength of about ± 5, ± 10, or ± 20 nm. In the examples, the exciting wavelength is of about 493 nm (± 10 nm), but it will be understood that the selection of the fluorescent dye used with the sample will determine the wavelength of the filter employed. The light first passes through a first lens 21 focused at a first aperture 22 having a diameter of 0.055" (1.4 mm) and then passed through a second lens 23 closer to the aperture than the first lens so that the beam is narrowed to pass through a second

aperture 24 having a diameter of 0.91" (23.1 mm). After passing through the second aperture 24 the beam is filtered by the first filter 25 to allow only light having the desired wavelength range to pass. After being filtered, the beam passes through a dichroic mirror 26 and then is reflected by a second mirror (27) 90° from its previous optical axis and then focused by a third lens 28 into the sample well 29 through its transparent bottom (see Figure 2b). Interaction of the exciting beam of light with fluorescent dye (in the examples, fluorescein isothiocyanate) causes fluoresced light to be emitted in all directions at wavelengths within a range determined by the dye selected for the particular application. The exciting beam passes out the top of the sample well 29. Only emitted light coming back through the transparent bottom of the sample well is to be measured and of that, only the portion having a predetermined wavelength. With the fluorescent dye used in the examples, the wavelength of the emitted light is about 530 (± nm). The emitted light is focused by the third lens 28 to form a beam of light which is reflected back by mirror 27 onto the dichroic mirror 26 which does not pass the light as before, but directs the light to the photodetector. (Note that by definition a dichroic mirror is able to pass light of predetermined wavelength(s), but will reflect light having wavelengths outside that range.) The reflected light first passes through a filter 31 which passes only light having the desired range of wavelengths, 530 (± 10 nm) in the examples below. After passing through a third aperture (32) of 1.22" (30.99 mm) diameter the light is focused through a fourth lens 33 to pass through a fourth aperture 34 of 0.1" (2.54 mm) before reaching the photodetector 30. The detector and its associated circuitry counts the photons received and reports the number of counts per second as the amount of fluoresced light detected (actually a known fraction of the photons actually received). The detected photon counts per second is correlated with the desired characterization of the sample being measured. Another method of measuring light is by charge integration. Either of these two methods could be used.

Alternative optical systems are shown in Figures 5 and 6. They are similar to the one shown in Figures 2a - d, but they differ in their construction and will provide an improved signal-to-noise ratio. The focusing of the exciting light in the sample well is substantially the same as in Figures 2b-c but the means for directing the exciting light to the sample well and the fluoresced light from the sample well to the photodetector differ from those shown in figure 2a. It should be noted that the disposition of each filter and

its associated aperture could be reversed if desired. In either instance a filtered and columated beam is produced.

In Figure 5, no mirror is used to direct the exciting light beam 90° from its original axis to reach the test well. The emitted light is reflected from the dichroic mirror through an annular aperture so that any exciting light that may be present tends to be blocked from reaching the photo detector.

In Figure 6, the dichroic mirror is omitted and replaced by a mirror containing a center aperture for passing the focused exciting light beam and reflecting the emitted light from the area of the mirror surrounding the center aperture. In other respects, the optical-system of Figure 6 is similar to that of Figure 5.

The signal-to-noise ratio should be optimized. That is, one wants to obtain the maximum output of the photodetector caused by the light emitted by the sample (the signal) with the least interference of light from other sources not related to the response of the fluorescent dye to the exciting light beam (the noise). As reported in the examples below, the signal-to-noise ratio (S/N) is defined as in terms of photodetector output in counts per second (CPS) $S/N = \frac{\text{Sample CPS} - \text{Blank CPS}}{\text{Blank CPS}}$. In this calculation, the fluorescence of a sample is compared to the fluorescence of a "blank", that is, a duplicate cell containing buffer solution, but no dye. The blank thus reports the native fluorescence of the sample cell and buffer solution when no dye is present.

The wavelength of the exciting light beam should be able to produce a strong response from the fluorescent dye. The dye used in the examples is excited by light in the range of about 440 to 520 nm and emits light in the range of about 500 to 580 nm. If the excitation wavelength is outside the excitation range, then the dye will not produce a useful response. The exciting light beam will normally be much stronger than the light produced by fluorescence and consequently, the excitation light should not return to the photodetector. For this reason, many previous optical systems provide for the exciting light to be deflected away after contacting the sample. In the present application, the exciting beam passes up through the window and the sample in the well and exits through the open top. Care is taken to limit the scattering of the exciting light and reflections or native fluorescence from the walls back through the bottom of the sample well. In one example below, it will be shown that intentionally scattering the light at the

top of the well is detrimental to the signal-to-noise ratio and consequently, would not be a preferred mode of operation. Another source of scattered light is the wall of the sample well. It has been found that when the exciting light beam is focused at the center of the sample and the cone produced by the converging light is narrowed so that it does not contact the well wall, either at the bottom or the top of the sample well, that scattering is reduced and the signal-to-noise ratio is improved. This also is shown in one of the examples below. Another aspect of the optical system which has been found to be important in obtaining the best performance is that excitation light which is scattered by the well wall or produced by the fluorescence of the well wall (the native fluorescence) should be prevented from reaching the photodetector. Such scattered light or native fluorescence are components of the "noise". This can be accomplished by proper selection of the lens which focuses the emitted light and the size of the aperture just adjacent to the photodetector. The annular apertures of Figures 5 and 6 also are intended to pass emitted light but to exclude scattered exciting light or other extraneous sources of light. In effect, the photodetector is only allowed to "see" light which has passed out from the center of the sample well and which has not been scattered from or produced by the walls of the sample well.

Of all the sources of "noise" which have been found, the native fluorescence of the bottom window is predominant. Thus, the bottom window should be made of a material which has a low response to the exciting light beam. Ideally, it would have none, but most transparent materials will have some response to the exciting light beam. Glass or quartz glass would be a good choice for low noise, but they are fragile and more difficult than plastic materials to fabricate into the plastic microplate. Other transparent materials which may have use are not entirely satisfactory, for reasons of performance or cost or ease of fabrication. The polystyrene often used in commercial microplates can be used although it has a higher native fluorescence than would be desired. However, it has been found that a satisfactory signal-to-noise ratio can be obtained provided that the thickness of the polystyrene is no greater than about 250 μm (0.010 inches). Polystyrene has other advantages which favor its use, as previously discussed above.

APPLICATIONS

Typically, a sample containing a fluorescent dye is excited with light having a wavelength suited to the dye selected. Fluorescence has many applications, as will be

vident to those skilled in the art. An example of such a use for the sample microplates of the invention is a fluorescence immunoassay, where a primary antibody is bound to the microplate well, and an antigen and second antibody tagged with a fluorescent dye are allowed to react with and bind to the primary antibody. Further examples include receptor binding, enzyme activity, ion channel, and nucleic acid assays, where one of the assay components is labeled with a fluorescent dye. Cell-based applications, in which cells are cultured directly in the microplate well, include cytotoxicity and reporter gene activity assays. Those skilled in the art will recognize that the range of possible applications is not limited to these examples.

Dyes which are of particular interest in assaying samples by fluorescence are those which emit light at wavelengths of about 400 to 700 nm. Chemical modification of these dyes, by attachment of so-called functional groups, allows them to be conjugated or chemically bound to the biological compound of interest. During a fluorescence assay, the light emitted by the dye is thus proportional to the amount of the compound to be measured. In one example of the invention, fluorescein isothiocyanate (FITC, which emits light in the range of about 500 to 580 nm) is conjugated to an antibody to a specific antigen. A second antigen-specific antibody is bound to the microplate well. When the fluorescently labeled antibody is mixed with the antigen in the well, a fluorescently labeled complex becomes bound to the well. After removing the unreacted labeled antibody, the fluorescence is measured and is directly proportional to the amount of antigen present in the sample. Examples of fluorescent dyes commonly used include, but are not limited to, rhodamine, BODIPY, Texas Red, and hydroxycoumarin. Those familiar with the art will recognize that a variety of fluorescent dyes are available for specific applications. Examples of such dyes are given in the following table.

Fluorochrome	Wavelengths (nm)	
	Excitation	Emission
NADH	Ug-11 (300-400)	460
Methylumbelliferone/(4-MUB, 4-MUP, 4-MUG)	360	460
AMC/amino-methylcoumarin	360	460
Hoechst Dye 33258/Bis-benzimide	360	460
DABA	400	530
AHOPhos™	485	570
Resorufin	460	570

Fluorochrome	Wavelengths (nm) Excitation	Emission
Acridine Orange/RNA	460	620
Acridine Orange/DNA	485	530
FITC (fluorescein-5-isothiocyanate)	485	530
Calcein	485	530
TOTO™	485	530
YOYO™	485	530
YO-PRO™	485	530
Propidium Iodide	530	620
Ethidium Bromide	530	620
Ethidium Homodimer	530	620
Alamar Blue	530	590
Rhodamine or Lissamine-rhodamine	530	580-590
XRITC (rhodamine-isothiocyanate)	530	620
Texas Red™	550	620
CY-5	610	670

Since the dyes vary in wavelength of light to which they respond (the excitation wavelength), the filter used with the light source will be selected to pass light within a narrow range of wavelengths suitable to excite the dye which is chosen. Correspondingly, the wavelength of the emitted light determines which filter is used before the photodetector so as to limit the extraneous light which is passed to within a narrow range of wavelengths.

Example 1

For FITC dye excited with light at 493 nm, examination of the potential range of wavelengths suggests that while 540 nm filters are useful, a 530 nm filter would be preferred. A monochromator based fluorometer (FluoroMax, Spex Industries Co, Inc.) was used to carry out a series of tests to examine the range of about 528 to 552 nm. The results are shown in Figures 3 and 4. In these tests, a slit width corresponding to a 10 nm bandwidth was swept over the range of emission wavelengths and the fluorescent signal in number of counts per second (CPS) was determined for samples which contained only a phosphate buffered saline solution (PBS) or a buffered solution containing 1.0 E^{-10} molar fluoroscein isothiocyanate dye (FITC). It can be seen from the figures that the buffer solution created very little "noise" and did not vary significantly with the wavelength being measured. ("Noise" refers to native fluorescence of the buffer

solution.) The dye responded very differently. It fluoresces, producing a signal that is much stronger at about 530 nm than at 550 nm. Thus, measuring the emitted light at the lower wavelength would be preferred. Another factor, not evident here, is that there appeared to be a Raman response by the water in the buffer at about 540 nm, making the choice of a 540 nm filter for these experiments somewhat less than optimum since water Raman emissions are not representative of the sample. Even so, as will be seen, it was possible to obtain satisfactory signal-to-noise ratios.

Figure 3 provides data obtained using a commercial quartz cuvette. It can be seen that the signal-to-noise ratio (S/N) appeared to be minimum at about 540 nm and that it was much improved at 530 nm. Figure 4 shows that when a commercial polystyrene cuvette was used that the signal-to-noise ratio was highest at about 530 nm and that no minimum was observed in the range measured.

Example 2

In the measuring system described, the exciting light enters at the bottom of the sample well and leaves from the top. This is desirable, since the exciting light is not scattered back from the sample well, provided that care is taken to avoid contact with the walls of the sample well. However, the light emitted from the sample will be lost in part since it will also exit from the top of the sample well. Consequently, an experiment was carried out in which a white colored light-diffusing cap was placed on top of a quartz cuvette and a comparison was made with the same cuvette without the cap. The results are shown in the table below. Z is the vertical position of the beam's focus in the sample well, with Z = 0 being below the well and Z = 24 being near the top of the well. The signals were measured when the sample well contained only the buffer solution and again, when it contained the buffer plus FITC dye. It can be seen that, although the signal (i.e. CPS of the emitted light) increased with the white diffusing cap in place as would be expected, the signal-to-noise ratio was decreased.

Table A

	BLANK CPS	
Z (mm)	Open Top	White Cap
0	5622	11431
6	5906	13816
12	6281	16298
18	6710	18529
24	7267	20329
DARK[1]	399	460
	FITC CPS	
Z (mm)	Open Top	White Cap
0	185322	225433
6	213287	263788
12	235665	269818
18	253497	322478
24	265736	340259
DARK[1]	416	482
	S/N RATIO	
Z (mm)	Open Top	White Cap
0	34.40	19.50
6	37.65	18.71
12	38.99	16.00
18	39.10	16.82
24	37.63	16.10

[1] DARK means residual CPS when the halogen lamp was turned off.

It was concluded that since the signal-to-noise ratio was about 144 % higher when the sample well was open at the top, it would be preferred to accept the somewhat lower intensity signal.

Example 3

As shown in Figure 2, the exciting beam is focused to a minimum beam diameter within the sample well. A series of experiments was carried out in which the minimum beam diameter was focused at various points within the sample well. Note that the beam is really conical; it has an hourglass-shape. When focused at the vertical midpoint of the sample well, the conical beam did not touch the walls of the well, either at the bottom or the top, as shown in Figure 2c. When focused between the vertical midpoint and the window, the beam was sufficiently wide toward the top of the well to contact the walls, causing reflection of some of the exciting light. When focused between the vertical midpoint and the top of the well, the beam was sufficiently wide near the bottom to

contact the walls, again causing reflection and scattering of some of the exciting light and possible excitation of fluorescence in the walls of the sample well. As shown by the results in the table below, the signal-to-noise ratio was highest when the beam was focused near the vertical midpoint of the sample well (Z = 6mm). In other words, avoiding reflecting and scattering of the exciting light is preferred if one wants to provide the best signal-to-noise ratio. In this example, measurements were made when the sample well contained four different volumes. It can be seen that varying the volume of the blank solution made little difference in the emitted light, while increasing the volume of the buffer solution containing dye increased the signal strength.

Table B

	BLANK CPS			
	VOLUME			
Z(mm)	**60 uL**	**125 uL**	**200 uL**	**250 uL**
0	25125	21032	22375	27647
6	22078	18883	19996	25860
12	23292	20032	20319	26517
18	27497	24322	23743	30062
24	41920	41810	39148	44454
DARK	460	477	479	482
	FITC CPS			
	VOLUME			
Z(mm)	**60 uL**	**125 uL**	**200 uL**	**250 uL**
0	88671	253271	507339	849215
6	92640	273817	544283	935784
12	96221	289563	571882	1001281
18	100341	299218	615598	1035781
24	111071	309221	678908	1046481
DARK	513	498	501	506
	S/N RATIO			
	VOLUME			
Z(mm)	**60 uL**	**125 uL**	**200 uL**	**250 uL**
0	2.57	11.30	22.15	30.24
6	3.26	13.85	26.86	35.85
12	3.19	13.78	27.80	37.44
18	2.69	11.53	25.44	34.00
24	1.67	6.47	16.54	22.79

Example 4

A series of experiments was carried out in a similar manner to those of Example 3, except that the detector's field of view of the emitted light was examined. It can be seen from Figure 2 that the emitted light is focused onto an iris (aperture 34) before

entering the photodetector, and that only a portion of the emitted light actually reaches the photodetector since some of it is blocked by the narrow iris. When the iris is narrowed so as to obscure any light reflected from or produced by fluorescence of the well wall the signal-to-noise ratio is seen to be improved. That is, it is preferred to limit the optical view of the photodetector to limit the emitted light to only that emerging from the center of the sample well. This will be evident from inspection of the data given in the table below.

Table C

VESSEL COLOR	VESSEL WINDOW	Aperture 34 = 0.250" (6.35 mm) FITC (cps)	PBS(cps)	S/N	Aperture 34 = 0.100" (2.54 mm) FITC(cps)	PBS(cps)	S/N	CHANGE: S/N	0.25" to 0.10" Gross FITC
White	Polystyrene	141437	65806	1.15	69040	25334	1.73	50.1%	-51.2%
White	Glass	82955	15530	4.34	50748	4021	11.62	167.7%	-38.8%
Black	Polystyrene	61268	18323	2.34	52657	14538	2.62	11.9%	-14.1%
Black	Glass	55827	8263	5.76	47533	2758	16.23	182.0%	-14.9%

Example 5

As experiments were carried out, it became apparent that the major contributor to the "noise" was the native fluorescence of the window at the bottom of the sample well. Data which demonstrate this conclusion are shown in the table below.

Table D

SAMPLE VESSEL	WINDOW MATERIAL	WINDOW THICKNESS (INCH) (μm)	EMPTY (CPS)	WATER (CPS)	PBS (CPS)
Black 96W uPlate(1)	polystyrene	.020 (508)	28442	22151	21910
Black 96W uPlate(1)	polystyrene	.005 (127)	12254	13496	11580
Black 96Well (2)	glass	.009 (229)	10493	10996	6292
White 96W uPlate(1)	polystyrene	.020 (508)	57371	55251	50317
White 96W Well(2)	glass	.009 (229)	17012	17165	14562
Cuvette(3)	quartz	.047 (1194)	3779	4532	3493
White 24W uPlate(4)	polystyrene	.024 (610)	38980	37369	35880
None	polystyrene	.024 610)	24672	N/A	N/A
None	glass	.009 (229)	12610	N/A	N/A
None	none	N/A	2879	N/A	N/A

(1) a 96 well microplate
(2) a 96 well microplate well cemented to a glass bottom
(3) commercial 1 cm x 1 cm x 3 cm quartz cuvette
(4) a 24 well microplate

The tests examined microplate wells with both black and white walls and cuvettes. These sample vessels were empty or contained water or PBS buffer solution. The window materials were polystyrene, glass, and quartz. In several cases ("None") no sample vessel was used, but only the window material was present.

Example 6

A summary of tests to determine the best signal-to-noise ratio is given in the table below. It can be seen that the optimum performance was obtained with a black microplate having a thin glass window and a narrow aperture (iris) adjacent to the photodetector. If polystyrene were to be used, it would be preferable to use as thin a window as possible to reduce the noise and thus to increase the signal-to-noise ratio.

Practical considerations would determine the thickness of a polystyrene window, but a window only 130 μm thick has been tested with good results.

Table E shows that the polystyrene window has significantly greater noise associated with its use. Clearly, glass provides a superior signal-to-noise ratio. However, as noted earlier, polystyrene has advantages which offset the additional noise and consequently, for many applications it would be preferred. To improve the signal-to-noise ratio it should be as thin as possible.

It can also be concluded from the data of Table E that a black-walled sample well would be preferred to improve the signal-to-noise ratio. It is believed that the superior results compared to the white-walled well were caused by a reduction in scattering of the excitation light or by lower native fluorescence.

Again, it can be seen that a glass window provides a better signal-to-noise ratio than is generally available with polystyrene.

In both tests with white and black walled test wells, it can be seen that the diameter of the aperture 34 (Fig 2a) affects the signal-to-noise ratio. Since the narrow aperture (0.1 inches) reduces the noise and increases the signal-to-noise ratio it may be concluded that the narrow aperture is blocking scattered excitation light to a greater extent than the emitted light.

The last three entries in Table E represent experiments done using the flat window material only, rather than a test well. No liquid sample was used since there was no test well to hold it. Instead, the exciting light passed through the flat window into air. Consequently, the light returning to the photodetector was scattered excitation light and native fluorescence of the window material itself. Again, the polystyrene window produced significant noise, the glass window less, and even with no window a certain amount of system noise was detected. Of course, since there was no test well, no measurements were made with dye present so a signal-to-noise-ratio could not be measured.

Table E

VESSEL	WINDOW	APERTURE 34 DIA	NET FITC SIGNAL (CPS)[1]	NOISE (CPS)	S/N
WHITE	POLY .020" THK	0.250" (6.25 mm)	77799	65806	1.182
WHITE	GLASS .009" THK	0.250" (6.25 mm)	69358	15530	4.466
WHITE	GLASS .009" THK	0.100"(2.54 mm)	48067	4021	11.954
BLACK	POLY .020" THK	0.250" (6.25 mm)	44176	18323	2.411
BLACK	POLY .005" THK	0.250" (6.25 mm)	53302	11580	4.603
BLACK	GLASS .009" THK	0.250" (6.25 mm)	48928	8263	5.921
BLACK	GLASS .009: THK	0.100" (2.54 mm)	46059	2758	16.700
		0			
NONE	POLY .024: THK	0.250" (6.25 mm)	None	24672	NA
NONE	GLASS .009" THK	0.250 (6.25 mm)	None	12610	NA
NONE	NONE	0.250" (6.25 mm)	None	2879	NA

[1] For this comparison the CPS were normalized to account for variations in the exciting light intensity.

WHAT IS CLAIMED IS:

1. In a microplate having a multiplicity of sample wells for holding a multiplicity of samples to be assayed by fluorescence and comprising an upper plate forming the side walls of said wells and a transparent lower plate forming the bottom of said wells and bands of opaque material to block the transmission of light between adjacent wells, the improvement comprising using as the material forming the transparent bottom of said wells material having a low native fluorescence and thereby improving the signal-to-noise ratio of light emitted by fluorescence of said samples and measured after passing through said transparent bottom.

2. The microplate of claim 1 wherein said transparent material is glass.

3. The microplate of claim 1 wherein said transparent material is polystyrene having a thickness no greater than about 250 μm (0.010 inches).

4. The microplate of claim 1 wherein said transparent material is quartz glass.

5. The micro plate of claim 1 wherein said side walls of said sample wells are opaque.

6. The microplate of claim 1, wherein the top of said sample wells is exposed to the atmosphere.

7. A method of assaying samples by fluorescence in a sample well comprising

(a) placing a sample capable of fluorescing when exposed to light in a sample well having opaque walls and a transparent bottom;

(b) focusing through the transparent bottom of said sample well a beam of light centered at a predetermined wavelength on said sample, thereby causing said sample to fluoresce;

(c) directing light emitted by fluorescence in step (b) from the bottom of said test well to a photodetector; and

(d) determining the desired property of said sample by the amount of light received by said photodetector.

8. The method of claim 7 wherein the transparent bottom of said sample well is polystyrene having a thickness no greater than about 250 μm (0.010 inches).

9. The method of claim 7 wherein the transparent bottom of said sample well is glass.

10. The method of claim 7 wherein the transparent bottom of said sample well is quartz glass.

11. The method of claim 7 wherein the top of said sample well is exposed to the atmosphere.

12. The method of claim 7 wherein the beam of light in step (b) is from a light source selected from the group consisting of tungsten halogen, xenon, xenon arc, and quartz halogen lamps and is limited to a range of about ± 5, ± 10, or ± 20 nm about said predetermined wavelength.

13. The method of claim 7 wherein the sample comprises a fluorescent dye capable of reacting with said beam of light to emit light by fluorescence.

14. The method of claim 13 wherein said fluorescent dye is selected from the group consisting of fluoroscein isothiocyanate, rhodamine, BODIPY, Texas Red, and hydroxy coumarin.

15. The method of claim 14 wherein said fluorescent dye is fluoroscein isothiocyanate.

16. The method of claim 7 wherein said beam of light is focused at the center of the sample well and does not impinge upon the well walls.

17. The method of claim 7 wherein said light emitted by fluorescence is focused on an aperture sized to prevent said photodetector from detecting scattered light or light emitted by native fluorescence from the walls of said test well.

18. The method of claim 7 wherein said light emitted by fluorescence is passed through an emission filter capable of passing only emitted light within a narrow range about a predetermined center wavelength.

19. An optical system for measuring by fluorescence a sample within a sample well having a transparent bottom comprising

(a) means for producing a columated beam of exciting light on a first optical axis and having a first wavelength;

(b) means for focusing the columated beam of exciting light of (a) through said transparent bottom onto said sample, thereby causing light having a second wavelength to be emitted by fluorescence;

(c) means for diverting emitted light of (b) through the transparent bottom from said sample onto a second optical axis;

(d) means for collecting and focusing said emitted light onto a photodetector for measuring the amount of said emitted light.

20. An instrument for measuring the light emitted by fluorescence of a sample in a sample well comprising:

(a) a light source;

(b) a first lens for focusing light produced by the light source of (a);

(c) a first aperture having a predetermined opening for passing light focused by the first lens of (b);

(d) a second lens for receiving light emerging from said first aperture and directing said light;

(e) a second aperture for passing light directed from said second lens of (d) and having a predetermined opening;

(f) a first optical filter for receiving light from said second aperture and capable of passing light within a predetermined wavelength range;

(g) a dichroic mirror capable of passing the filtered light of (f) and reflecting emitted light from (i);

(h) a mirror for directing said filtered light of (f) passed through the dichroic mirror of (g) to a sample well and for directing light emitted by fluorescence from within said sample well;

(i) a third lens for focusing said directed light of (h) onto a predetermined location within said sample well and for directing light emitted by

fluorescence from within said sample well to said mirror of (h) and thereafter to said dichroic mirror of (g);

(j) a second optical filter for receiving reflected emitted light from said dichroic mirror of (g) and capable of passing the emitted light within a predetermined wavelength range;

(k) a third aperture having a predetermined opening for passing said filtered emitted light of (j);

(l) a fourth lens for receiving said filtered emitted light of (j) passed through said third aperture and focusing said light;

(m) a fourth aperture having a predetermined opening for passing said focused light of (l); and

(n) a photodetector for measuring said filtered emitted light of (j) passed through said fourth aperture of (m).

21. An instrument for measuring the light emitted by fluorescence of a sample in a sample well comprising

(a) a light source;

(b) a first lens for focusing light produced by the light source of (a);

(c) a first aperture having a predetermined opening for passing light focused by the first lens of (b);

(d) a second lens for receiving light emerging from said first aperture of (c) and directing said light;

(e) a second aperture for receiving light from said second lens of (d) and having a predetermined opening;

(f) a first optical filter for receiving light passed through said second aperture of (e) and capable of passing light within a predetermined wavelength range;

(g) a dichroic mirror capable of passing the light passed through said second aperture of (e) and reflecting the emitted light from said sample wells;

(h) a third lens for focusing the light passed through said dichroic mirror of (g) onto a predetermined location within said sample well and for directing light emitted by fluorescence within said sample well to said dichroic mirror of (g);

(i) a third aperture having an annular opening for passing reflected emitted light from said dichroic mirror of (g);

(j) a second optical filter for receiving said reflected emitted light from said third aperture of (i), and capable of passing the emitted light within a predetermined wavelength range;

(k) a fourth lens for receiving the filtered emitted light passed through the second optical filter of (j) and the third aperture of (i) and focusing said light;

(l) a fourth aperture having a predetermined opening for passing the light focused through said fourth lens of (k); and

(m) a photodetector for measuring said filtered emitted light passed through said fourth aperture of (l).

22. An instrument for measuring the light emitted by fluorescence of a sample in a sample well comprising

(a) a light source;

(b) a first lens for focusing light produced by the light source of (a);

(c) a first aperture having a predetermined opening for passing light focused by the first lens of (b);

(d) a second lens for receiving light emerging from said first aperture of (c) and directing said light;

(e) a second aperture for receiving light from said second lens of (d) and having a predetermined opening;

(f) a first optical filter for receiving light passed through second aperture of (e) and capable of passing light within a predetermined wavelength range;

(g) a mirror having an opening through which the light passed through said second aperture of (e) can pass, said mirror positioned to reflect emitted light from said sample well;

(h) a third lens for focusing the light passed through the opening in said mirror of (g) onto a predetermined location within said sample well and for directing light emitted by fluorescence within said sample well to said mirror of (g);

(i) a third aperture having an annular opening for passing reflected emitted light from said mirror of (g);

(j) a second optical filter for receiving said reflected light of (i), from said third aperture of (i) and capable of passing the emitted light within a predetermined wavelength range;

(k) a fourth lens for receiving the filtered emitted light passed through the second optical filter of (j) and the third aperture of (i) and focusing said light;

(l) a fourth aperture having a predetermined opening for passing the light focused through said fourth lens of (k); and

(m) a photodetector for measuring said filtered emitted light passed through said fourth aperture of (l).

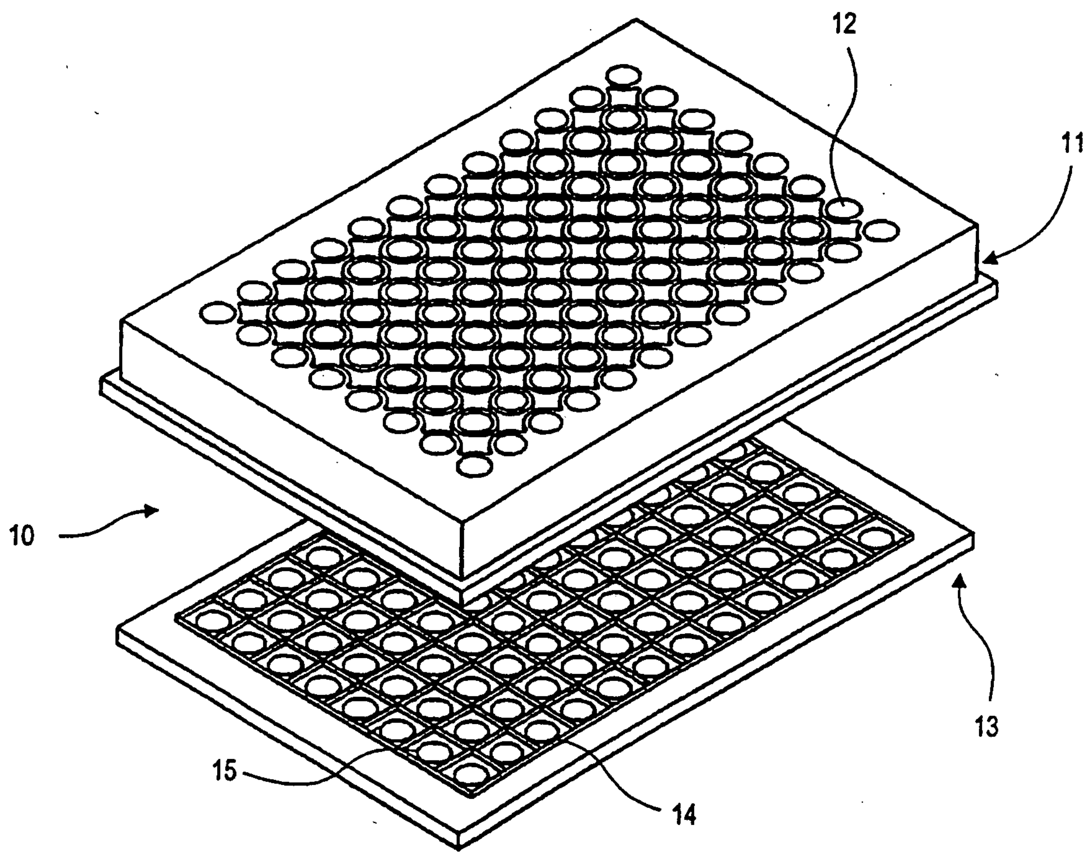

FIG. 1